

Molecular phylogenetics and the classification of honey bee viruses

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Summary. We present the phylogenetic relationships of several picorna-like RNA viruses found in honey bees, with respect to 13 additional plant and animal positive-strand RNA viruses. Most of the honey bee viruses fall into an unnamed family of insect RNA viruses typified by the *Drosophila* C virus. Different bee viruses are broadly distributed within this group, suggesting either that the ability to infect honey bees has evolved multiple times, or that these viruses are generalistic in their abilities to infect insect hosts. At least one major change in gene order has occurred among the bee viruses, based on their phylogenetic affiliations. At the amino-acid level, the bee viruses differed by 15–28% at three conserved loci. Most differed by greater than 50% at the RNA level, indicating that sequence-based methods for bee virus identification must be tailored to at least three different virus clades independently.

Introduction

In addition to producing honey and other valuable hive products, honey bees (*Apis mellifera*), provide important pollinator services worldwide, with a net economic value in the tens of billions of dollars. Parasites and pathogens can strongly impact both feral and domesticated honey bee populations [22]. The most visible threats to honey bees, and arguably the most important, are parasitic mites in the genus *Varroa*. Nevertheless, the impact of parasitic mites can be heightened by the effects of other honey bee parasites and pathogens, including viruses [7, 8, 15].

Thanks to several decades of study, a considerable database exists for the serological and physical identification of honey bee viruses (reviewed by [3, 6]) and for determining the efficacy of several virus species experimentally [2, 4]. Despite these advances, the impacts of specific virus taxa on honey bee populations have been notoriously difficult to assess [7, 31]. We feel that these difficulties have arisen from two major barriers. First, it has proved difficult to determine the presence of viruses in bees from the field, except in those (apparently rare) cases when individual bees manifest diagnostic physiological and behavioral

symptoms. Consequently, estimates for the frequencies of latent viruses are incomplete. Genetic assays for key viruses will provide better estimates of virus presence in honey bee populations, data that can be used to assess the frequency and severity of their lethal and sublethal effects.

A second hurdle for determining the importance of bee viruses involves virus taxonomy. The challenge of identifying bee viruses has been met, on some level, through the use of serological assays based on antibodies developed from particular virus isolates [1]. One weakness of these assays rests with the investment required to develop precise antibodies for each member of the broad family of bee viruses. To be current and inclusive of all possible viruses, serological assays require the continuous development of antibodies for known and emergent virus strains, an effort that is unlikely to occur for honey bees. Finally, honey bee viruses, like disease agents in humans [21], can be mistyped because of cross-reactivity in serological assays.

Molecular-genetic techniques offer a means of precise and sensitive detection of viruses, and one that can provide direct comparisons across a diversity of viruses. Stoltz et al. [33] developed the first primers for a honey bee virus, using a 417-nucleotide region of the RNA-dependent RNA polymerase (RdRp) gene in the ‘Kashmir bee virus’, a picorna-like RNA virus found in honey bees worldwide. Subsequent RNA sequence analyses for this locus show that it can be used to distinguish both between and within recognized Kashmir bee virus strains [13]. As additional RNA-level sequences of bee viruses become available, including sequences of entire virus genomes [11], it will be crucial to place these viruses into a phylogenetic framework based on their evolutionary relationships. Here we present a preliminary analysis of the phylogenetic relationships of all bee viruses for which sequence data are available. We also propose general guidelines for the efficient development and use of genome sequences for efforts aimed at reducing the impact of viruses on honey bee populations. These guidelines should be generally useful in providing benchmarks for virus taxa that are at an early stage of formal taxonomic description.

Materials and methods

Collection and characterization of Kashmir bee virus isolates

Virus samples from bees putatively infected with Kashmir bee virus (as assayed by serological tests; [14]) were collected from colonies of *A. mellifera* in Beltsville, MD. RNA extraction and amplification by reverse-transcriptase PCR were carried out as described [13]. A 683-base pair section of the RNA-dependent RNA polymerase gene was amplified in two sections, using primer pair KBV1 (5'-GATGAACGTCGACCTATTGA-3') and KBV2 (5'-TGTGGGTTGGCTATGAGTCA-3'), and primer pair KBVUSD129 (5'-ATGAAGTGTCTATTGGAACG-3') and 80812B11.R (5'-TTCGAACCCTCGCCTCCA-ACTCC-3'). DNA sequencing was conducted for each strand of each PCR product on four virus isolates from the United States, one from Canada and one from Australia [13], using standard dye-primer reactions and an Applied Biosystems 377 Automated sequencer. Consensus RNA sequences were determined for each sequenced strand after alignment using the software program Omega 1.1 (Oxford Molecular Products, Campbell, CA).

Collection of additional sequences from genomic databases

Amino-acid and RNA sequences from the genomes of 16 additional picorna-like virus genomes were collected from the Genbank and Swissprot databases through the use of the BLASTX search algorithms, at the web site maintained by the U.S. National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>). A search of the Genbank database on July 8, 1999, revealed several hundred significant Genbank matches with the protein inferred by the 683-nucleotide RNA sequence of the Kashmir bee virus. All of these matching protein sequences were derived from psRNA viruses, and most had been confirmed as the product of the RNA-dependent RNA polymerase (RdRp) gene in these viruses, strongly suggesting that the Kashmir bee virus RNA sequences were indeed from this gene. The protein sequence from one additional bee virus, sacbrood virus (accession number AF092924; [11]), was retrieved in this way. Two more honey bee virus proteins were uncovered via the Entrez search engine, also at the NCBI site. One of these consisted of a partial sequence for the helicase gene of black queen cell virus (accession number AF125252; R. C. Ghosh, Genbank submission), and the inferred protein from this sequence. The final sequence entry reflected a segment of one of the capsid proteins from a European isolate of acute bee paralysis virus (accession number AF126050; R. C. Ghosh, Genbank submission).

For the phylogenetic analyses, we chose additional representative amino-acid sequences from 13 other psRNA viruses that have been sequenced in full. This group includes four members of the *Picornaviridae* family, six picorna-like insect viruses, and three plant viruses in the family *Sequiviridae*. Accession numbers for these sequences, along with data on genome size and order, are presented in Table 1. We collated amino-acid sequences for the RNA polymerase, helicase and homologous coat proteins for each virus taxon where possible (sequences for KBV were restricted to the RdRp, for black queen cell virus to the helicase protein and, for acute bee paralysis virus to one of the structural proteins).

Sequence alignment and phylogenetic analyses

Amino-acid sequences were inferred from the RNA sequences, then were aligned using Omega 1.1. Amino-acid sequences for the helicase, RdRp and capsid protein genes were aligned using the Gonnet series scoring algorithm, with an open-gap penalty of 10.00 and an interior-gap penalty of 0.5 per residue site (Omega 1.1 User Guide, Oxford Molecular, 1998). Sequence alignments used for each locus are available upon request.

Aligned sequences were imported into the phylogenetic analysis program PAUP 4.02b [34]. Phylogenetic relationships were inferred for each gene independently (for all taxa for which amino-acid sequences were available at a particular gene) using a heuristic parsimony search algorithm based on tree bisection-reconnection resampling [34]. Interior gaps predicted by the sequence alignments were weighed the same as individual amino-acid changes. For each gene, the sequence for *Hepatitis A* was used as an outgroup, since sequence data are available for this virus at each of the genes assayed, and since *Hepatitis A* is presumed to fall outside the clade encompassing all of the insect and plant viruses we used in the analyses. The statistical strength of taxonomic groups predicted by the phylogenetic trees was inferred by bootstrap analyses ($n = 10,000$ replicates). Finally, a combined-data phylogeny for these taxa, including all of the bee viruses, was derived by placing both the RdRp and helicase proteins into a single alignment. For these analyses, interior gaps were again scored as new states, while characters not sequenced in some of the taxa (including entire genes in the case of Kashmir bee virus and black queen cell virus) were, naturally, scored as 'missing' data. This phylogenetic tree was subjected to bootstrap analyses as above ($n = 10,000$ replicates).

Table 1. Virus taxa used for phylogenetic analyses, with code names, host type (IN = insect, PL = plant, MA = mammal), Genbank sequence accession number, genome position of RNA-dependent RNA polymerase (5' = on upstream side of capsid proteins), genome size, and sequence reference

Virus	Code	Host	Access.#	RdRp	Size (# bases)	Sequence reference
Kashmir bee	KBV	IN	AF177935, AF034541	5'	9,500 ^a	This paper, [13]
Acute bee paralysis	AcuteBee	IN	AAD33287	5'	~9,500	Ghosh, et al. (GB)
Cricket paralysis	Crickparal	IN	P13418	5'	~9,500	[18]
<i>Drosophila C</i>	DrosophC	IN	AF014388	5'	9264	[17]
<i>Rhopalosiphum padi</i>	Rhopad	IN	AF022937	5'	10,011	[23]
Black queen cell	Blkqueen	IN	AF125252	5'	~9,500	Ghosh et al. (GB)
<i>Himetobi P</i>	Himetobip	IN	AB017037	5'	9275	Nakashima (GB)
<i>Plauti stali</i>	Plautist	IN	AB006531	5'	8797	[30]
Maize chlorotic	Maizchlor	PL	U67839	3'	11,832	[29]
Rice tungro spherical	Ricetung	PL	RTUPOLYP	3'	12,226	[32]
Parsnip yellow fleck	Parsnipy	PL	D14066	3'	9871	[28]
Sacbrood bee	Sacbrood	IN	AF092924	3'	8832	[11]
Infectious flacherie	Silkflach	IN	AB000906	3'	9650	[16]
Coxsackie	Coxsackie	MA	M88483	3'	7399	[20]
Poliomyelitis 2	Polio2	MA	PIPOLS2	3'	7439	[36]
Mengo	Mengo	MA	L22089	3'	7761	[25]
Hepatitis A	HepatitA	MA	GNNYHB	3'	7477	[27]

GB Sequence in Genbank public database

^aThe genome size of KBV was estimated to be 9,500 bases by [1] and 10,500 bases by [33]. Viruses of the honey bee are in bold type

Results

Homology of the KBV sequences and other RNA polymerase genes

Both the Genbank searches and our phylogenetic analyses place Kashmir bee virus, black queen cell virus, acute bee paralysis virus and sacbrood virus into a broad clade of plant and animal picorna-like viruses. For the RdRp protein, Kashmir bee virus and sacbrood showed key amino-acid motifs that have been used to characterize the picorna-like viruses (Table 2; [19]). Similarly, the aligned sequences for the helicase genes of both black queen cell virus and sacbrood (Table 3) showed diagnostic amino-acid residues for this group. Finally, the structural-protein sequences of both acute bee paralysis virus and sacbrood virus matched at motifs used to define VP3 and homologues from the picorna-like viruses (data not shown; the nomenclature of this capsid protein is inconsistent across different virus families since naming was based on protein size rather than sequence homology, as discussed in Ghosh et al. [11]).

Despite sharing several sequence patterns at the amino-acid level (Tables 2, 3), the RNA sequences of the bee viruses were strongly divergent. Within Kashmir bee virus, the only honey bee virus to date for which sequence data allow intraspecific comparisons, sequences differed by 5% between the Australian

Table 2. Alignment of *A. mellifera* viruses with conserved amino-acid motifs found across psRNA viruses, RNA-dependent RNA polymerase locus

Virus	I	II	III
AustKBV	???????PIEK (7)	KTRVFSNGPMDFSIAFRMYLGFIAHLMENR- (6)	IGTNVYSQDW (16)
UsKBV	???????RPIEK (7)	KTRVFSNGPMDFSIAFRMYLGFIAHLMENR- (6)	IGTNVYSQDW (16)
DrosophC	TLKDERRDIAK (7)	KTRVFSAGPQHVFVAFRQYFLPFAAWLMHNR- (6)	VGTVNYSSDW (16)
Rhopad	TLKDQKIAIAK (7)	KTRLFSAAPMHYAIALRKVCAPFVAHLSRMR- (6)	VGVPNPFSEW (16)
Himetobip	TLKDERK---P (8)	KTRMFSAACPLDYLIACKMYFGGVVSLQKSR- (6)	VGTVNYSYDW (15)
Plautist	TLKDERK---A (8)	KTRLFSASPLPYLILCRMYLQGGVSRIRGK- (6)	VGTPNYSDDW (16)
Maizchlor	CPKDERRKLSK (8)	ATRTFTTILPPEINILFRQYFGDFAAMIMTNR- (6)	VGINPENMEW (16)
Ricetung	CLKDERRKLAK (8)	ATRTFTTILSPEVNILFRQYFGDFAAMVMSTR- (6)	VGINPESMEW (16)
Parsnipy	CPKDERRALDK (8)	KTRLFSILPVEFNMHARRLFDFNVFVMANR- (6)	VGINPHSREW (16)
Sacbrood	TLKDERKLPEK (8)	GTRVFCNPPIDYIVSMRQYMHFVAAFMEQR- (6)	VGINVQSTEW (16)
Silkflach	HLKDELRPSEK (8)	GTRVFSVPPELVLVNSRRFLLPFMDAFQSFP- (6)	IGLNPNSGDW (16)
Coxsackie	HVKDELRSEK (7)	KSRLIEASSLNDVAMRQTFGNLYKTFHLNPG (6)	VGCDPDL-FW (13)
Polio2	YVKDELRSEK (7)	KSRLIEASSLNDVAMRMAFGNLYAAFHKNPG (6)	VGCDPDL-FW (11)
Mengo	FLKDELRPIEK (7)	KTRIVDVPPFEHCILGRQLLGKFASKFQTQPG (6)	IGCDPDV-HW (15)
HepatitA	CPKDELRLPEK (7)	KTRAIDACPLDYTILCRMYWGPAISYFHLNPG (6)	IGIDPDC-QW (16)

Virus	IV	V	VI	VII
AustKBV	GDFSTFDGSLN (51)	???????????????????? (##)	???????????? (##)	????????????
UsKBV	GDFSTFDGSLN (51)	SGNPATTPLNCFINSMLRMCF (21)	LVSYGDDNVI (##)	????????????
DrosophC	GDFGNFDGSLV (55)	SGNPFTVIIINCLYNSIIMRLSW (22)	LITYGDDNVL (41)	EDIFFLKRKF
Rhopad	GDYSNFDGSLP (74)	SGCPVTAPLNSIVNQMALVYCW (20)	SVFYGDDFVM (42)	PEVNFLLKRAF
Himetobip	GDFEGFDSSQL (55)	SGHFLTAIINSIFVLISFNSVW (19)	IVAYGDDHIV (42)	NEVSYLKRKF
Plautist	GDFASYDSSQE (58)	SGHFLTSIINSIFVNIAMCYAF (22)	IVTYGDDHVI (42)	EEVTFIKRSF
Maizchlor	GDYSKFDGIGD (51)	SGFAMTVIFNSFVNYYLAMAW (23)	VVVYGDDNIV (44)	TSVSFLKRRW
Ricetung	GDYSKFDGIGS (51)	SGFAMTVIFNSFVNYYFMALAW (23)	IVAYGDDNVV (44)	TKMSFLKRGF
Parsnipy	GDFANFDGMFH (49)	SGFPMTVIFNSFINLFYLSAW (23)	ACVYGDDNIV (44)	LEFDLKRHF
Sacbrood	IDYSNFGPGFN (53)	SGAPITVVINTLVNIIYIFVAW (21)	LFCYGDDLIM (41)	LNSTFLKHGF
Silkflach	MDYKNYSDAIP (55)	AGHPMTSVVNSVNLILMNYMW (16)	IIVMGDDVVI (43)	DKFEFLSRGF
Coxsackie	FDYSGYDASLS (46)	SGCSGTSIFNSMINNIIIRTL (13)	MIAYGDDVIA (36)	TNVTFLKRYF
Polio2	FDYTG YDASLS (45)	SGCSGTSIFNSMINNIIIRTL (13)	MIAYGDDVIA (36)	ENVTFLLKRF
Mengo	VDYSNFDSTHS (48)	SGCAATSMLNTIMNIIIRAGL (13)	VLSYGDDLIV (37)	EDVVFLLKRF
HepatitA	LDFSAFDASLS (48)	SGSPCTALLNSIINNVLNLYVF (16)	ILCYGDDVLI (42)	SELTFLKRSF

Amino-acid residues conserved across all virus taxa shown in bold. Numbers refer to amino-acid residues between each motif. Motifs numbered according to [19]

Table 3. Alignment of *A. mellifera* viruses with conserved amino-acid motifs of other psRNA viruses, helicase locus. Numbers refer to amino-acid residues between each motif

Virus	I	II	III	IV
DrosophC	RMRPICLWLVGESGVGKTEMV (38)	QKIVIIYDD (30)	MAALHDKN-TFSAEELLYTTN (12)	AFNRM
Rhopad	RTEPFVIVWFSGASGNGKTGLS (34)	QEYIVYDD (30)	MASLLDKNNTFAEPKLIICLTSN (12)	AVSRRI
Blkqueen	???????????????????? (##)	???????? (##)	?ASIEEKANTVFQSKVILCSSN (12)	ALLRRF
Himetobip	RNPPVVIYLHGGSGVGKSTLT (40)	QLVTVFDD (30)	MANLSDKASTNFTSKIIICSSN (12)	ALYRRF
Plautist	RPPPVSLLLLGGTGRGKTTVT (44)	QLITVFDD (30)	MANLEDKNNTWFRSSVILASSN (16)	ALLRRF
Maizchlor	RIDPFYICLTGPPGVGKSTVA (34)	EPVIIYDD (28)	MAAVEEKG-RHCLSXYLVACTN (13)	AYYRRI
Ricetung	RIDPLHVCMLGAPGVGKSTIA (37)	EPVILYDD (28)	MAAVEDKG-KHCTSKYVFSCTN (13)	AYYRRR
Parsnipy	RVDPFHVSLYGSFVGKSFVM (34)	QTAVKCDD (28)	MADLANKG-RTFTSKYIFSTTN (13)	AFMRRR
Sacbrood	RYEPFVICIEGPAGIGKSEIV (36)	QPVVVYDD (28)	MAHLEEK-IRGNPLIVILLCN (15)	AIYRRR
Silkflach	RFEPFVVWIFGPRGVGKSTLL (38)	QPIVLYDD (29)	KPRIEEKE-SLMTSVIVGIASN (12)	AMDRRR
Coxsackie	RIEPVCLLLHGSFGAGKSVAT (30)	QAVVIMDD (25)	MAALEEKG-ILFTSPFVLASTN (12)	ALARRF
Polio2	RIEPVCLLVHGSFGTGKSVAT (30)	QGVVIMDD (25)	MASLEEKG-ILFTSNYVLASTN (12)	ALARRF
Mengo	RCEPVVIVLRGDAGQGKSLSS (32)	QFAAIMDD (25)	MASLERKG-TPFTSQLVVATTN (12)	AVERRI
HepatitA	RCEPVVCYLYGKRGGKSLTS (34)	QLVCIIDD (25)	MASLEEKG-RHFSSPFIIATSN (12)	AIDRRL

and North America isolates, and by 1–2% within the North American isolates. KBV showed only 50% sequence similarity at the RNA level (339/683 nucleotides) with *Drosophila C* virus, putatively the next most closely related species. Similarity between the Kashmir bee and sacbrood virus RNA sequences was extremely low, precluding an accurate sequence alignment between these taxa.

Even at the amino-acid level, pairwise sequence similarities across species were low. In contrast to the North American and Australian isolates of KBV ($\geq 98\%$ sequence identity), sacbrood virus showed only 28% amino-acid identity to KBV at the RNA-dependent polymerase locus (62/225). Sacbrood showed 23% identity to black queen cell virus at the helicase gene and 15% identity to acute bee paralysis virus at the protein gene VP3 (21/139).

Acute bee paralysis virus showed 29% (39/132) identity to *Drosophila C* virus, 28% (38/133) identity to cricket paralysis virus, 29% (36/122) to *Plauti stali* virus, and 31% (65/208) to *Himetobi P*. For comparison, cricket paralysis virus was 71% identical to *Drosophila C* virus at the same section of the capsid protein.

Phylogenetic relationships among the picorna-like viruses

Kashmir bee virus was placed into a clade of several insect-specific viruses typified by the *Drosophila C* virus (Fig. 1A). In contrast, black queen cell virus is consistently allied with an adjacent clade, composed of two viruses, *Himetobi P* and *Plauti stali* virus, that infect members of the order Hemiptera (Fig. 1B, Fig. 2). In the combined-data analyses, bootstrap values supporting the split between Kashmir bee virus and its clade, versus black queen cell virus, were strong

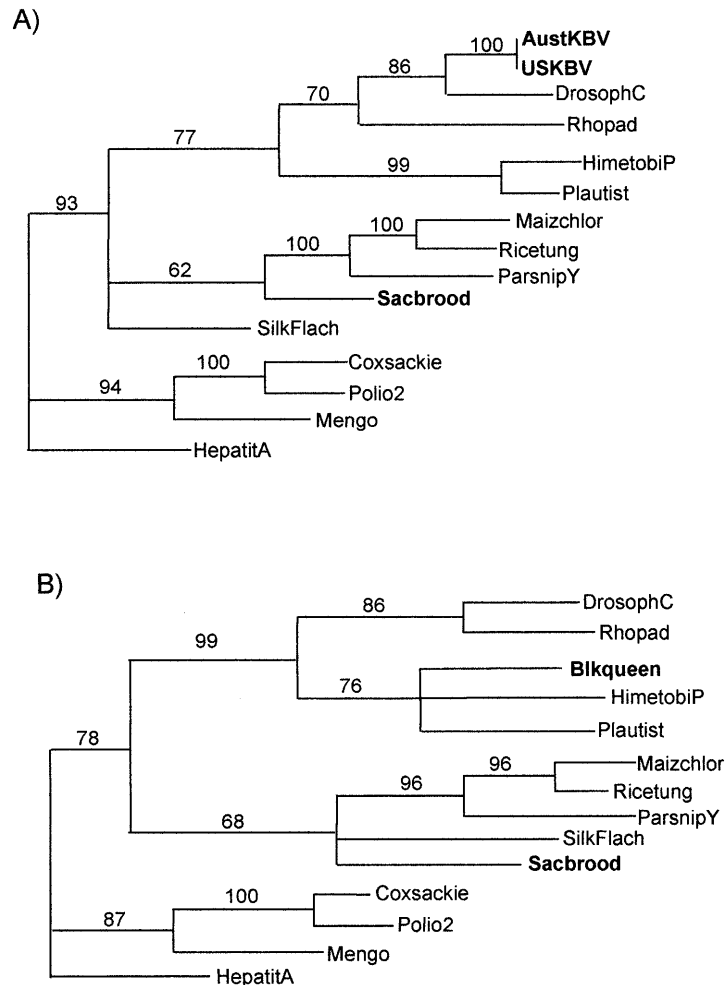


Fig. 1. Phylogenetic relationships among positive strand RNA viruses, based on amino-acid sequences for **A** RNA-dependent RNA polymerase and **B** helicase. Viruses found in honey bees shown in bold. Numbers refer to bootstrap values for particular taxonomic groupings

(Fig. 2). Phylogenetic analyses based on structural proteins (not shown) suggest that both the honey bee acute bee paralysis virus and cricket paralysis virus should be included in the *Drosophila C* virus group. This phylogenetic hypothesis is also supported by the sequence-identity data presented above.

Discussion

Our analyses strongly suggest that the ability to infect honey bees has evolved multiple times in psRNA viruses. Indeed, the amino-acid similarity between sacbrood virus and three other bee viruses was no higher than that between sacbrood and members from two families of plant viruses. Sacbrood was relatively close, in amino-acid sequence, to infectious flacherie virus, an infectious agent found in silkworms and other Lepidoptera [16]. The more distant relationship between the

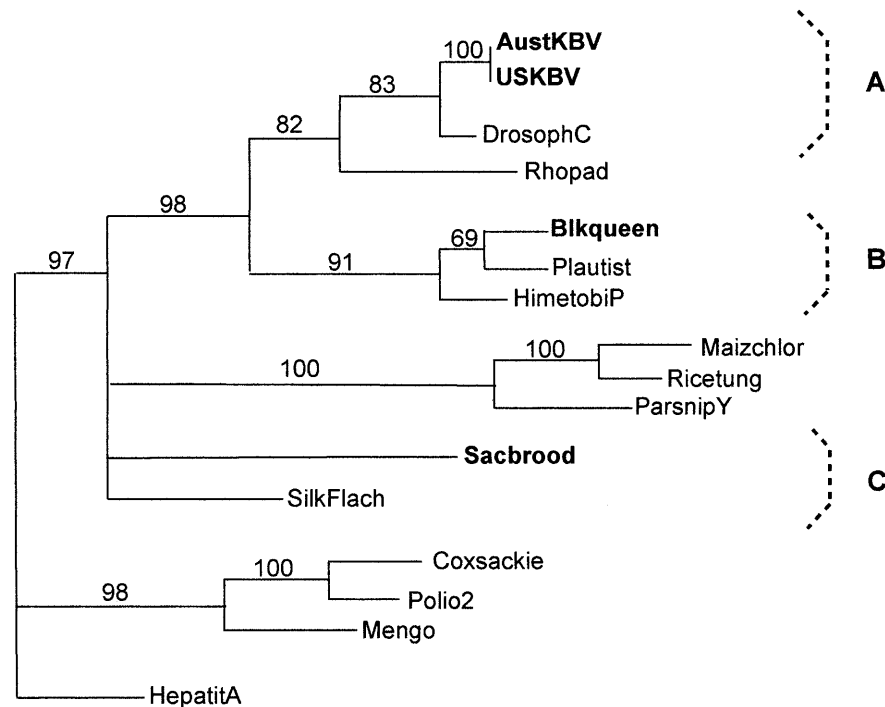


Fig. 2. Combined-data phylogeny for honey bee viruses and other positive-strand RNA viruses, generated using amino-acid data from the RNA-dependent RNA polymerase and helicase sequences. Numbers refer to bootstrap values. Letters A, B, and C refer to the minimum number of clades in which a honey bee virus is found

‘sacbrood-IFV’ group and the clade containing Kashmir bee virus (KBV), black queen cell virus, and acute bee paralysis virus is corroborated by differences in gene order inferred for these groups (Table 1, see also Ghosh et al. [11]). The structural, or capsid, proteins are found near the start of the linear genome in sacbrood, infectious flacherie virus, and most other psRNA viruses from plant and vertebrates. In contrast, Kashmir bee, acute bee paralysis and black queen cell viruses appear to fall into a growing clade of insect psRNA viruses typified by the *Drosophila C* [17] and cricket paralysis viruses [9], whose structural proteins fall at the downstream (3′) end of their genome. Within this group, black queen cell virus was allied with viruses isolated from the hemipterans *Plauti stali* and *Himetobi sp.*, while Kashmir bee virus and acute bee paralysis virus were paired with *Drosophila C* virus and a virus isolated from the aphid *Rhopalosiphum padi*.

The phylogenetic diversity of the bee viruses has two major implications for the study and control of bee viruses. First, control strategies targeting specific bee viruses should benefit from field and laboratory data collected from their respective relatives. For example, it is likely that insights gained from the control of the infectious flacherie virus e.g., [38] in silkworms will apply best to sacbrood virus, while data showing the means of dispersal, infection and replication by

Drosophila C virus [35] and its relatives will be most applicable to KBV and acute bee paralysis viruses. Further, control strategies for black queen cell virus might best start from extensive evidence gathered for the closely related *Himetobi* P and *Plauti stali* [24] viruses.

Second, great genetic distances between sacbrood and the other bee viruses, and indeed between each of the bee viruses, suggest challenges to the development of sequence-based assays for viral presence. At the nucleic-acid level, honey bee viruses show only minor sequence similarities. Since current molecular-genetic techniques rely on close matches between samples and the oligonucleotide primers used to identify them [12, 33], it is implausible to design a universal sequence-level marker that can detect members of the broad spectrum of bee viruses. Instead, primers are needed for each of several bee virus groups. These primers should be designed from the RNA sequences of the most highly conserved regions known from the virus genomes, such as conserved regions of the RdRp gene, after alignment of all known sequences in a group.

Ongoing efforts to sequence entire viral genomes will greatly speed the molecular-genetic characterization of bee viruses and their phenotypes. Recently, Ghosh et al. [11] presented the first complete sequence for a honey bee virus genome (sacbrood virus). Genomic sequence analyses offer the best opportunity to determine the specific amino-acid residues that confer pathogenic traits on bee viruses. To this end, future sequencing projects should include both virulent and avirulent strains of virus species. In the barley stripe virus, an important pathogen on grains in the United States, genetic analyses were used to pinpoint a single amino-acid change that had a tremendous effect on the pathogenicity of this virus [39]. Sequence-level analyses of viruses have also been used in the development of virus-resistant plants [37], and should prove useful for similar breeding programs involving honey bees.

Serological and genetic techniques for identifying bee viruses should complement one another fully, following a widespread strategy used to describe mammalian viruses [10, 26]. A preliminary serological characterization of virus isolates from bees can group viruses into broad functional groups (e.g., ‘sacbrood’ versus ‘Kashmir bee’ versus ‘black queen cell’). These assays then can be followed by sequence analyses, using oligonucleotide primers that are compatible with all members of the appropriate virus group. Following RNA sequencing and alignment with known virus genomes, primer pairs can be designed to specifically amplify genome segments from individual virus strains. With these strain-specific primers in hand, it should be possible to rapidly, and with great sensitivity, screen for viruses in potentially infected bees or potential vectors (including the mite *Varroa jacobsoni*; [5, 8, 40]). As a bridge between serological characterization and strain-specific genetic tests, phylogenetic analyses should be generally useful for the study and control of emergent or taxonomically ambiguous viruses.

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